A CHROMATOGRAPHIC STUDY OF THE BODY MUCUS OF SEVERAL IOWA FISHES

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INTRODUCTION

More and more modern taxonomists are finding chromatography a useful tool. It has been used to separate or attempt to separate species of various groups of organisms and presents a new means of showing taxonomic relationships in still other groups. Many present taxonomic methods are tedious and difficult. They might be reinforced or even replaced by advanced chromatographic techniques. It is hoped that the results of this study can contribute to the growth of these methods.

The first studies using chromatography to distinguish between strains of organisms were carried out by Buzzati-Traverso and Rechnitzer. Buzzati-Traverso (1953) used paper partition chromatography on tissues of different strains of Drosophila melanogaster and certain plants, and found that each strain gave a constant distinctive pattern. Buzzati-Traverso and Rechnitzer (1953) worked with fresh tissues of several species of fish, with results showing constant and easily recognizable differences between species. Results also seemed to indicate that the closer the taxonomic position of the species studied, the greater the similarity of their chromatographic patterns. Snails were the next group to be explored. Fresh tissue extracts of the foot muscle of seven species of land snails were studied with chromatogra-

phy. Each species was discernable from the others (Kirk, Main and Beyer, 1953). Wright, Harris and Claugher (1957), working with land snails and certain aquatic groups, improved on previous methods by the use of advanced chromatography apparatus. Free amino acids of certain coelenterates, arthropods, molluscs and echinoderms were found to give characteristic patterns for individual species (Simpson, Allen and Awapara, 1959). Micks (1954) used paper chromatography for a taxonomic study of mosquitos, finding consistent differences in their ninhydrin stained chromatograms. Numerous other studies using chromatography as a taxonomic tool have been completed since these initial ones.

Some of the most tedious and time consuming methods known to taxonomists are required to distinguish between species of fish. Conventional methods include: counting scales, observing pharyngeal teeth, making various measurements and observing coloration and body shape. When working with small or immature specimens, and groups of closely related species, the traditional methods of the fish taxonomists have limitations; therefore new methods are constantly being sought. Chromatography is a relatively new method and may provide taxonomists a more objective procedure.

Several studies attempting to use chromatographic methods in fish taxonomy have been reported. The first, that of Buzzati-Traverso and Rechnitzer (1953), showed that

definite differences could be demonstrated in the chromatograms of fluorescent and ninhydrin-positive substances occurring in fresh tissue extracts of various species. Consistent chromatographic patterns were reported among members of the same species. Comparison of chromatograms indicated that the closer the taxonomic relationship between species, as reflected in systematics based on other factors, the greater the similarity in the chromatograms. Matsumoto (1964) using fresh muscle and fluid squeezed from muscle tissue of larval and post-larval tunas found inconsistencies among chromatograms. Using similar methods Viswanthan and Pillai (1956) showed differences between species of Sardinella, Leiognathus, Caronz Atherina and Hemirhamphus georgy. Carlson (1961) was able to show quantitative, but not qualitative, differences in the bound amino acids in muscle tissue in three genera of lampreys.

Serological techniques have also been used to seek biochemical differences between species of fish. O'Rourke (1959) found a close relationship among the antisera of the cod (Gadus callavias), the haddock (G. aeglifinus) and the pollack (G. polbeneinus) with the pollack and haddock being closely related and the cod equally related to both of them.

In 1959 the idea of the possible species specificity of fish body mucus was suggested by Barry and O'Rourke. They reported chromatographic differences in the mucus pro-

duced by Gadus pollachine, Gadus irrens, Sebastus marinies and Sebasteis mentella. However, each comparison required a specific solvent system. Huntsman (1964) studied the body mucus of several species of Carpiodes. Chromatograms failed to show distinguishable differences between species. He did show differences between certain genera. Even where taxonomists have failed to find characteristic chromatographic patterns for individual species, all have felt that there was great potential in this area.

The body mucus of fish is composed of a combination of mucin and water. The mucin is produced by simple epidermal mucous cells, which vary in number, kind and size in different species of fish. These cells are derived from the basal layer of the epidermis (Van Oosten, J., 1957). Little work has been done on the chemical nature of fish body mucus. It was found to be composed mainly of a simple protein component which contains little or no carbohydrate. Some fish mucus also contains small amounts of carbohydrate and variable quantities of one or more glycoproteins. These glycoproteins contain: hexosamine, galactose, fucose and sialic acid (Enomoto, N. and Y. Tomiyasu, 1960; 1961a,b; Enomoto, N., T. Izuimi and Y. Tomiyasu, 1961; Enomoto, N., T. Nagao and Y. Tomiyasu, 1960; 1961; and Wessler and Werner, 1957).

Fish body mucus has a number of functions. It reduces friction in water, allowing greater speed with less

energy expenditure (Van Oosten, J., 1957). It also protects the fish from attack by parasites, fungus and bacteria. The antibiotic and toxic activity of fish body mucus has been demonstrated in Grammistis sexlieatus. Mucus from this species inhibits growth of Escherichia coli and is lethal to Fundulus heteroclitus (Liguori, Ruggieri, Baslow, Stempien and Nigrelli, 1963). Serum protein antigens have been demonstrated in fish body mucus. Their function is not known. O'Rourke (1961) hypothesized that species specific antigens are responsible for the ability of parasites to find their hosts. Another function of body mucus is the precipitation of foreign toxic compounds, especially on the gill filaments. If the turbidity or the concentration of toxic materials is too great the mucus covering coagulates and suffocates the fish (Van Oosten, J., 1957; Doudroof and Kratz, 1953; Nikolsky, 1963 and Westfall, 1945). By lubricating the surface, mucus prevents irritating substances from coming in contact with the body, and also plays a role in osmoregulation (Van Oosten, J., 1957). Abegg (1949) suggested that the body surface of a fish is altered when subjected to salt solutions. He says the mucus is either dissolved or coagulated. Jakowska (1963) reviewed the possible functions of fish body mucus and included temporary shelter, attachment of young, support for developing eggs and feeding of young.

Mucus is produced in varying amounts depending on con-

ditions and species. Strahan (1959) found that Myxine glutinosa, e.g., produced unusually large amounts. As a general rule mucus production is correlated with scalation; the higher the degree of scalation the less mucus is produced.

Fish mucus has proved to be complex chemically, and plays a complex role in the life of the organism. It is such an integral part of the fish that it is subject to all the selective pressures which favor differentiation among species. For this reason, mucus may be a very important subject for chromatographic techniques, contributing to both taxonomy and biological theory and reflecting functional lines of adaptation.

METHODS AND MATERIALS

To determine the species specificity of fish body mucus by chromatography it is essential that a suitable system be found. Both thin layer and instantaneous thin layer chromatography were used in this study. These systems have the same basic principles as all chromatography methods. There are three components; a mobile phase, a stationary phase, and a sample. Separation occurs by the combination of a retardation of the sample by the stationary phase and a moving of the sample by the mobile phase. There are four main mechanisms of retardation of the stationary phase. The first is adsorption or the electrostatic attraction of the

sample by active sites on the adsorbent. The second is partition, which deals with the solubility of the sample in a stationary liquid phase held by the adsorbent. Next is ion-exchange, with small inorganic ions attached to the adsorbent. Finally, there is molecular sieving, differential exclusion of the sample by porous materials because of molecular shape and size. All four mechanisms are usually involved in any chromatographic separation; however one is usually dominant (Gelman, 1968). Thin layer and instantaneous thin layer methods provide rapid and precise separations of small quantities of substances.

Two chromatographic systems were tried in preliminary studies. The first was Eastman Kodak's sandwich development chamber with their thin layer silica gel (type 6060) medium with fluorescent indicator. The 20cm. x 20cm. sheets were cut into strips 2cm. x 20cm. and activated by heating in an oven at 100°C for 20 minutes. The strips were spotted with fresh mucus, scraped directly off the fish with a microspatula (Huntsman, 1964). The spotted strips were dried in a dessicator before runs were made. Three solvent systems were used: chloroform/methanol/acetic/acid/phenol (20:10:1:1,v/v/v/v), chloroform/95% ethanol/acetic acid/phenol (20:10:1:1,v/v/v/v) and chloroform/methanol/17% ammonia (2:1:1, v/v/v). None of the runs achieved good separations and it was difficult to work with the medium due to the flaking off

of the adsorbent. The sheets had to be handled carefully and therefore, were judged not readily adaptable to this work.

The second method tried was Gelman's thin layer chromatography chamber with Gelman's S and SG instantaneous thin layer media. The procedure was the same for both. The medium was either activated (heated at 110°C for 45 minutes) or left inactivated. Mucus scraped directly from a fish with a microspatula was spotted on 5cm. x 20cm. strips. The sample was then placed in a dessicator for a minimum of five minutes. The chromatography chamber was prepared by placing a "solvent saturation pad" soaked with the solvent to be used. The prepared chromatogram was then put in the chamber and allowed to equilibrate for at least 20 minutes. After this time the solvent used was added to the chamber by the use of a thistle tube (Gelman, 1967). Solvent systems and lengths of runs were varied.

Gelman's S type instantaneous thin layer medium was developed specifically for the separation of carbohydrates, leaf chloroplasts and dyes (Gelman, 1967). This medium was used in an attempt to obtain specific patterns of possible carbohydrate components of the body mucus. Chromatograms were sprayed with aniline phthalate and heated at 110°C for development. Solvent systems used with the type S paper were: 95% ethanol/pyridine/water (8:2:1,v/v/v), 1-butanol/pyridine/water (2:6:3,v/v/v), amyl alcohol/pyridine/water

(4:4:1,v/v/v), and 1-butanol/acetic acid/diethyl ether/water (9:6:3:1,v/v/v/v). None showed the presence of separable quantities of carbohydrates.

Gelman's SG type instantaneous thin layer medium is the most versatile and sensitive type available (Gelman, 1967). This medium was used in an attempt to obtain specific patterns of the amino acid and other possible components of body mucus. Chromatograms were sprayed with ninhydrin and heated for development. Solvents used were: chloroform/methanol/acetic acid/phenol (20:10:1:1,v/v/v/v), 1-butanol/acetic acid/water (100:22:50,v/v/v), 95% ethanol/water (7:3, v/v), 95% ethanol/30% ammonia (7:3,v/v), n-propanol/water (7:3,v/v), n-propanol/30% ammonia (8:2,v/v), and 1-butanol/acetic acid/water (100:30:50,v/v/v). Again the lengths of the runs in which these solvents were used, were varied. Of the solvents used, n-propanol/30% ammonia (7:3,v/v) and n-propanol/30% ammonia (8:2,v/v) separated mucus components best. Separations of components were accomplished with runs of approximately 10cm. in length.

The SG type instantaneous thin layer medium was also used in an attempt to separate the possible carbohydrate components of the mucus. Solvents used were: amyl alcohol/pyridine/water (4:4:1,v/v/v), 95% ethanol/pyridine/water (8:2:1,v/v/v), 1-butanol acetic acid/diethyl ether/water (9:6:3:

1,v/v/v/v), pyridine/1-butanol/water (2:6:3,v/v/v). None of these attempts proved satisfactory.

Four species in the family Cyprinidae and one species in the family Percidae were used in this study. Notropis dorsalis dorsalis (central bigmouth shiner), Notropis deliciosus (sand shiner), Hybognathus nuchalis nuchalis (silvery minnow) and Pimephales notatus (bluntnose minnow) represent the family Cyprinidae and Etheostoma nigrum nigrum (central johnny darter) represents the Percidae (Eddy, 1957 and Harlan and Everett, 1951). These species were chosen because of their availability and taxonomic relationships to one another. Specimens of various sizes and sexes were represented in the samples. The fish were seined from Little Beaver Creek and Big Creek in Polk County, Iowa. They were then transported to the laboratory in styrofoam boxes in the water from which they were collected. Mucus was gently scraped from the fish, as in preliminary studies. Mucus was obtained with some difficulty due to the fact that scales were easily scraped off with the mucus. This was eliminated as much as possible by being especially careful and attempting to remove any scales from the mucus.

Gelman's SG instantaneous thin layer medium with n-propanol/30% ammonia (8:2,v/v) proved the best system for separations and therefore was used in final analysis. Mucus scraped from the specimens was spotted on activated 5cm. x 20cm.

strips of medium. Two samples were placed on each strip. Rubber gloves were worn when handling fishes, eliminating possible hand proteins from interfering with trials. Runs were approximately 10cm. in length and lasted approximately 40 minutes. The chromatograms were developed by spraying with ninhydrin and heating. The developed chromatograms were compared by observing the number of spots and the Rf values of these spots. By examining these values for the various species, evidence of species specificity was sought.

Rf value is defined as the migration distance of the substance over the distance traveled by the solvent front (Block, Durrum and Zweig, 1964). Rf values are affected by temperature, humidity and other factors and for this reason trials were made in the shortest time span possible (Block, Durrum and Zweig, 1964 and Geiss and Schlitt, 1963).

RESULTS

Work was limited to ascending chromatography due to the construction of the apparatus used. A total of 130 chromatograms were developed in this study: 26 for Notropis dorsalis dorsalis, 48 for Notropis deliciosus, 18 for Pimephales notatus, 26 for Hybognathus nuchalis nuchalis and 12 for Etheostoma nigrum nigrum. Various combinations of these species were worked with at separate times. No differences were observed between chromatograms of the same fish seined

from the two sources. Differences in patterns were found in chromatograms of the same species run at separate times. Figure I compares chromatograms of Notropis deliciosus run approximately two weeks apart. Chromatogram A represents 16 specimens and chromatogram B, 10 specimens. As one can see A has a spot with .56-.61 as its Rf value and B a spot with .41-.46 as its Rf value; otherwise they are similar. This is an example of differences in spot location which occurred. The number of spots also varied in certain comparisons of this type. These variations were great enough to prevent comparison of data collected during the separate runs. Thus, all comparisons in this work were made between specimens studied during the same 12 hour period. Matsumoto (1960) working with fresh tissue and Huntsman (1964) working with mucus also found inconsistencies in patterns of the same species worked with at separate times. Differences include number and location of spots. A variance in location of spots could partially be attributed to factors which affect Rf values. These have been discussed previously. The difference in the number of spots might have been the effect of humidity, temperature and atmospheric constituents on the color development in the ninhydrin stained chromatograms (Carlson, 1961 and Huntsman, 1964).

A comparison of chromatograms of two closely related species showed similarities in both number and location of

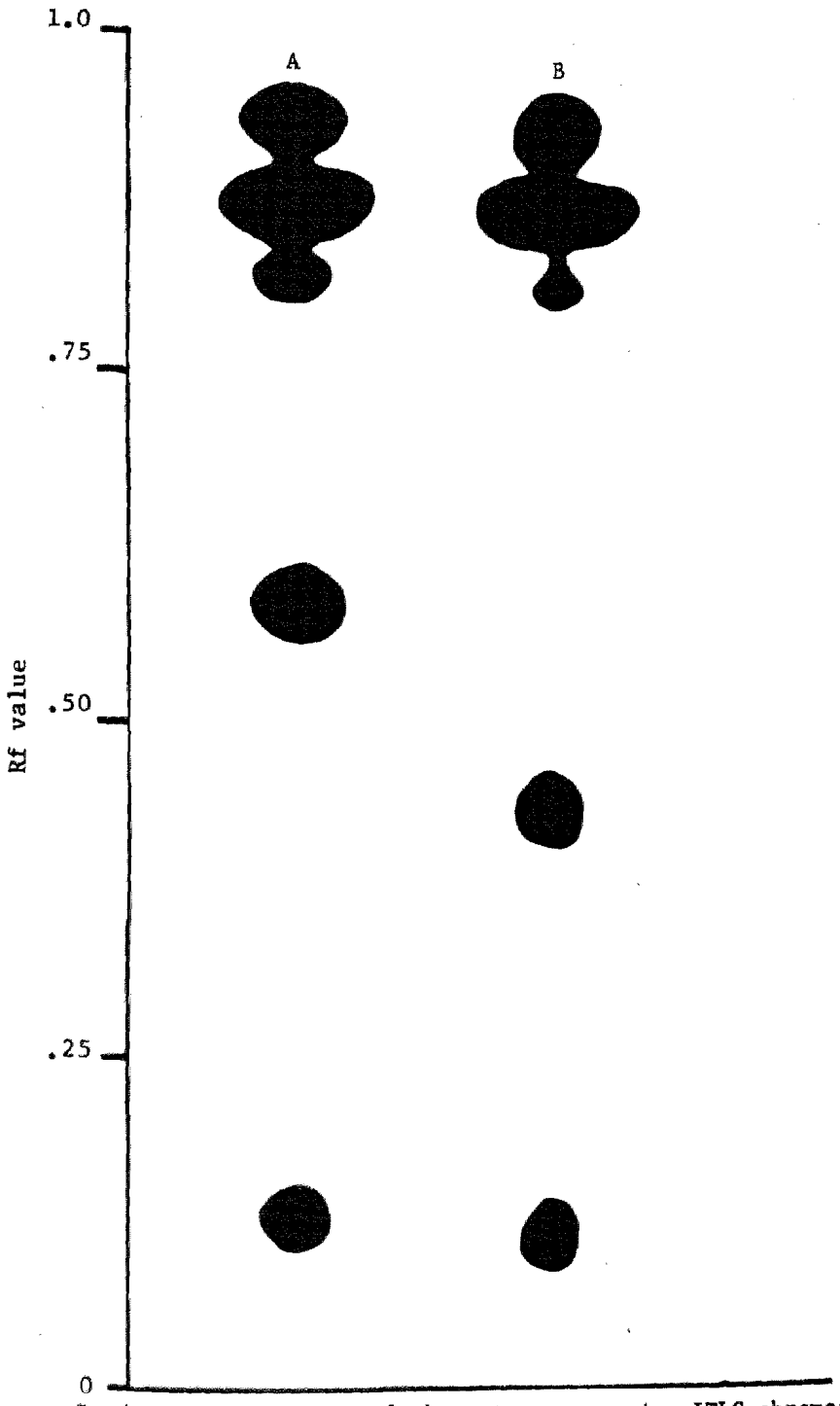


Figure I A representation of chromatograms, using ITLC chromatography, of Notropis deliciosus run approximately two weeks apart in n-propanol and 30% ammonia (8:2 v/v) for 10cm. Chromatograms were developed with ninhydrin

spots and also in general appearance. Figure II represents chromatograms of Notropis dorsalis dorsalis and Notropis deliciosus. A total of 23 specimens of N. dorsalis dorsalis and 16 of N. deliciosus are represented. The chromatograms of these two species were essentially indistinguishable from one another. Differences were seen in the per cent occurrence of the spots between the two species. Figure III shows the per cent occurrence of the various spots. The greatest variance occurred in the spot with the lowest Rf value. This spot occurred in 87.0% of the Notropis dorsalis dorsalis specimens and in only 6.25% of the Notropis deliciosus specimens. Thus it was impossible, except through differences in the per cent of occurrence to demonstrate species specificity of fish body mucus.

Comparisons between the genera of one family and between families themselves were made next. Figure IV shows chromatograms of the family Percidae (Etheostoma nigrum nigrum) and three genera of the family Cyprinidae (Notropis deliciosus, Hybognathus nuchalis nuchalis and Pimephales notatus). The chromatograms are representative of 12 Etheostoma nigrum nigrum specimens, 10 Notropis deliciosus specimens, 10 Hybognathus nuchalis nuchalis specimens and 10 Pimephales notatus specimens. Distinct differences were found between chromatograms of the two families. They involved both the number and location of spots. The chromatogram of Etheostoma nigrum

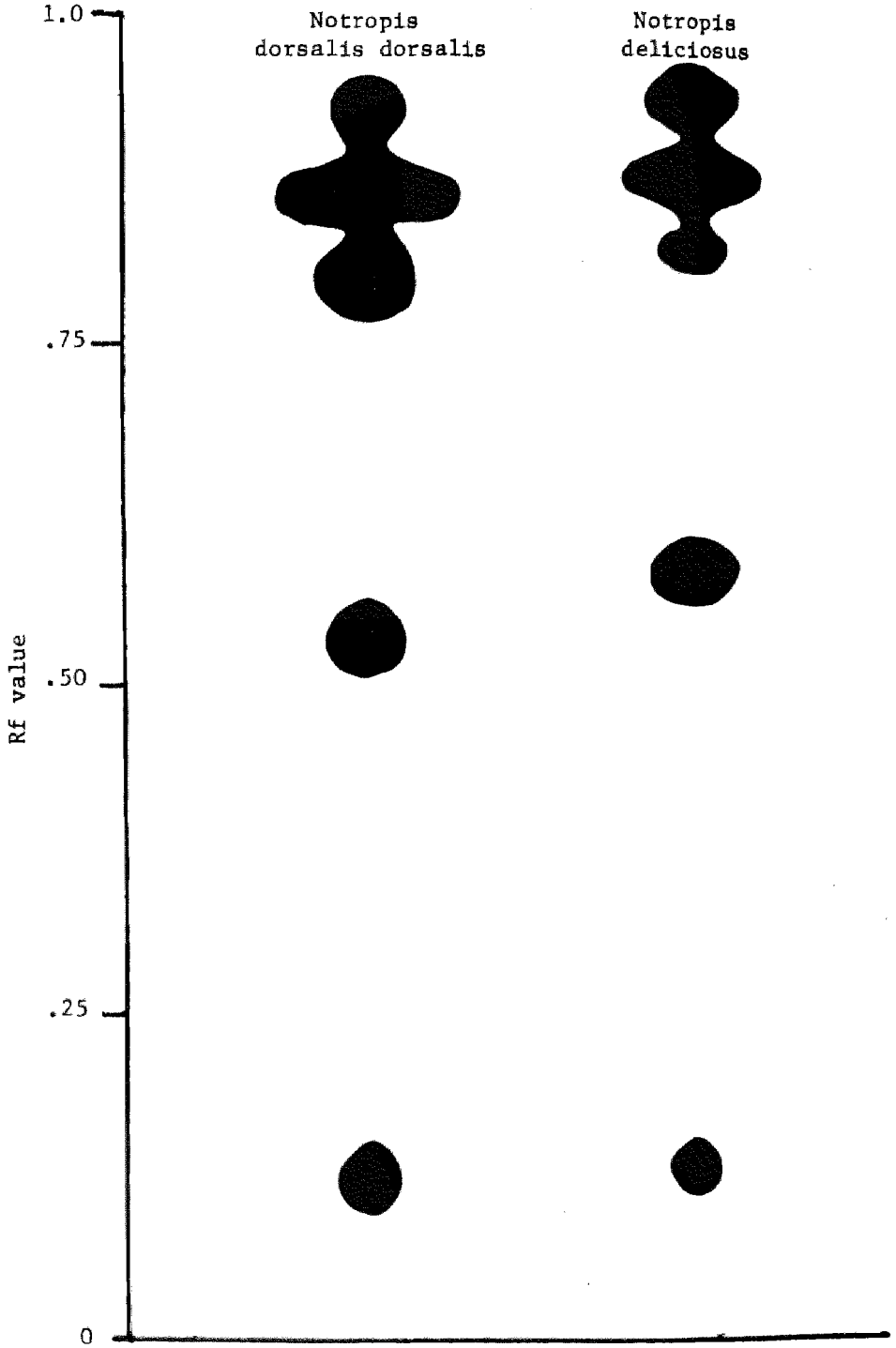
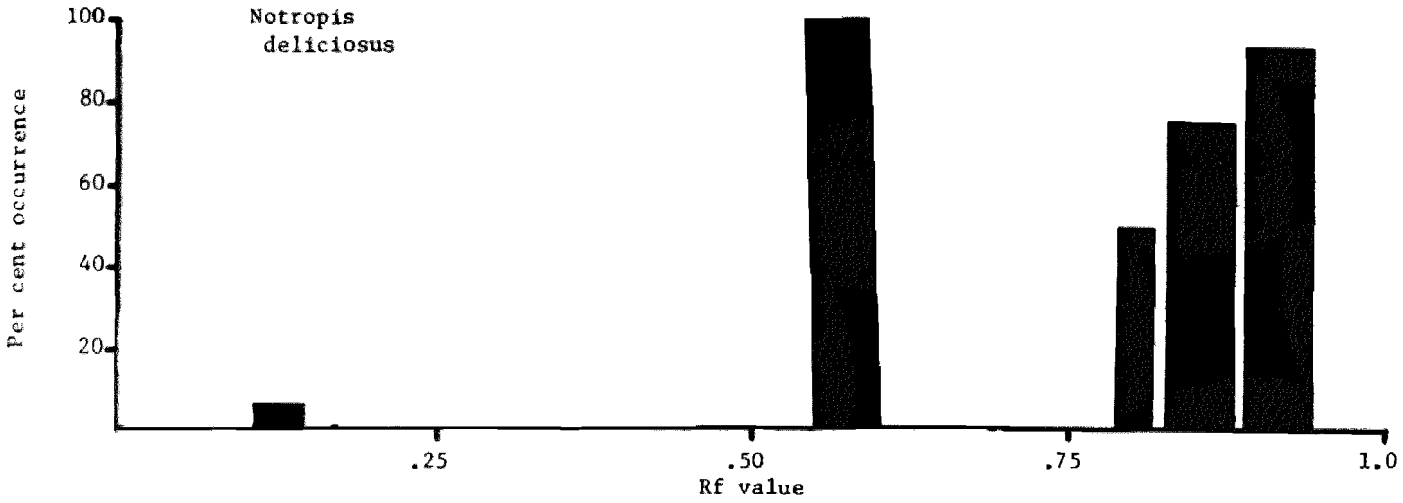


Figure II A representation of chromatograms, using ITLC chromatography, of Notropis deliciosus and Notropis dorsalis dorsalis run in n-propanol and 30% ammonia (8:2 v/v) for 10cm. Chromatograms were developed with ninhydrin.



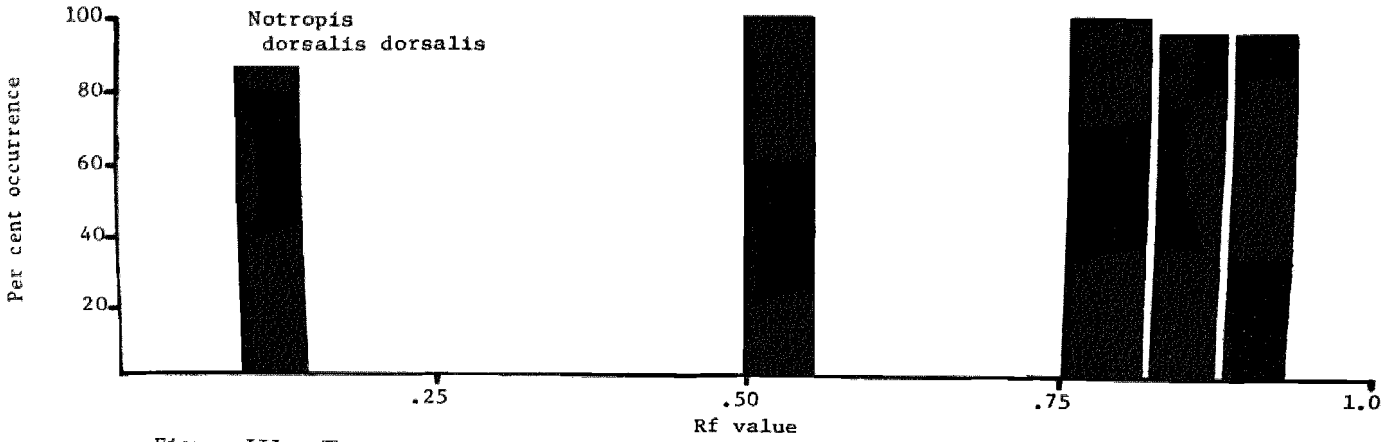


Figure III The per cent occurrence values of the spots found on chromatograms of Notropis deliciosus and Notropis dorsalis dorsalis.

nigrum contained 8 spots, while the species representing the family Cyprinidae had 5-6 spots. Differences between the members of the family Cyprinidae were fewer and less distinct. Hybognathus nuchalis nuchalis chromatograms had one more spot than did the other two species. This spot had an Rf value of .54-.60. The other spots of Hybognathus nuchalis nuchalis and those of Pimephales notatus and Notropis deliciosus were the same in number and location. The general appearance of the chromatograms, however were different. The per cent occurrence of comparable spots among the chromatograms differed. These differences are shown in figure V. It must be stated that the chromatograms of the three genera of the family Cyprinidae showed more similarities than differences. All chromatograms were examined with ultraviolet light, none exhibited fluorescence. No attempt was made to identify the substances separated.

DISCUSSION

Due to the short time needed to complete a run and the ease of handling, ITLC seemed to present an ideal method for determining the possible species specificity of fish body mucus. These two advantages also enhance the use of such a method in taxonomy. The use of fish body mucus instead of fresh tissue also is advantageous, because it eliminates the need of killing the specimen.

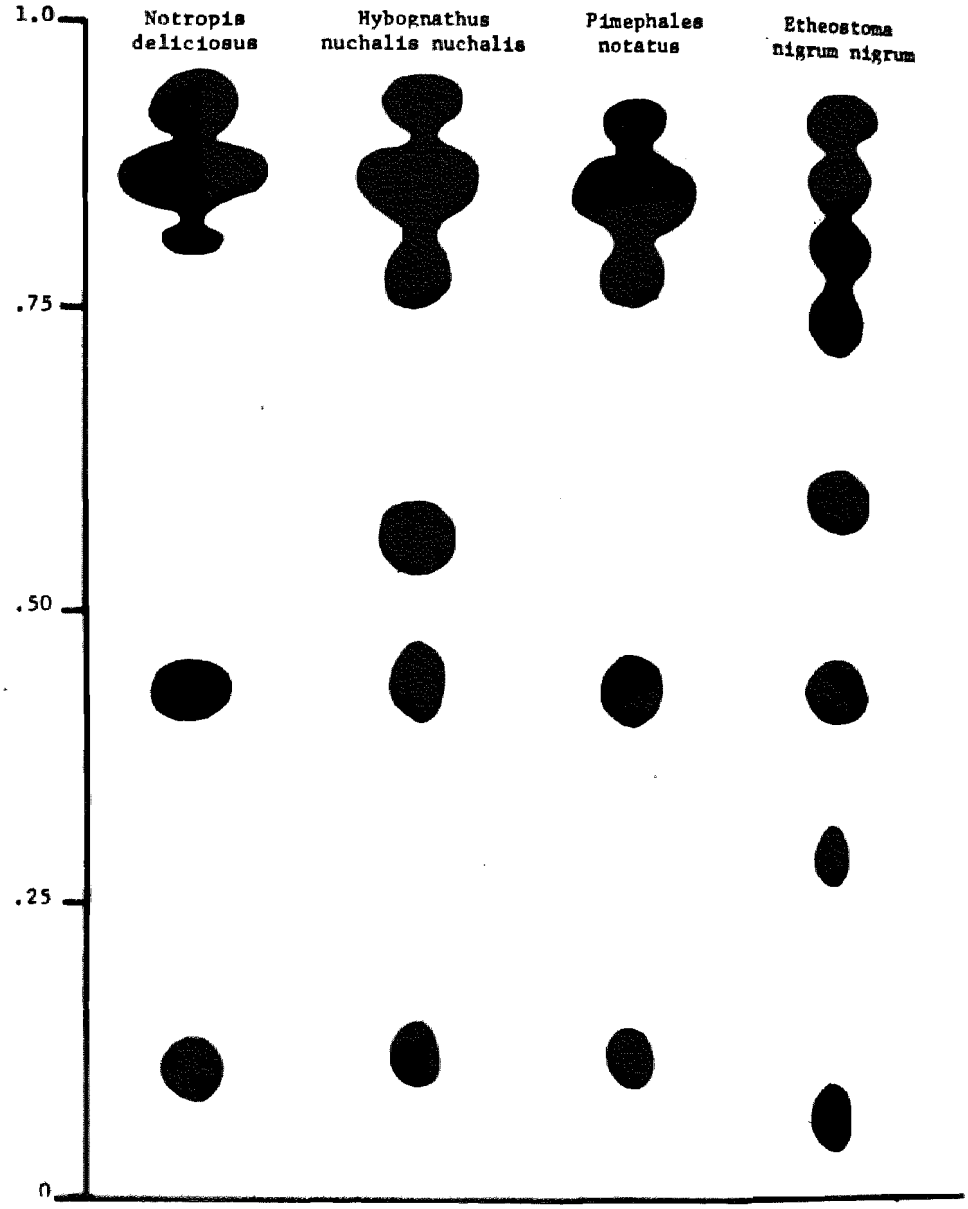


Figure IV A representation of chromatograms, using ITLC chromatography, of Etheostoma nigrum nigrum, Notropis deliciosus, Pimephales notatus and Hybognathus nuchalis nuchalis run in n-propanol and 30% ammonia (3:2 v/v) for 10cm. Chromatograms were developed with ninhydrin.

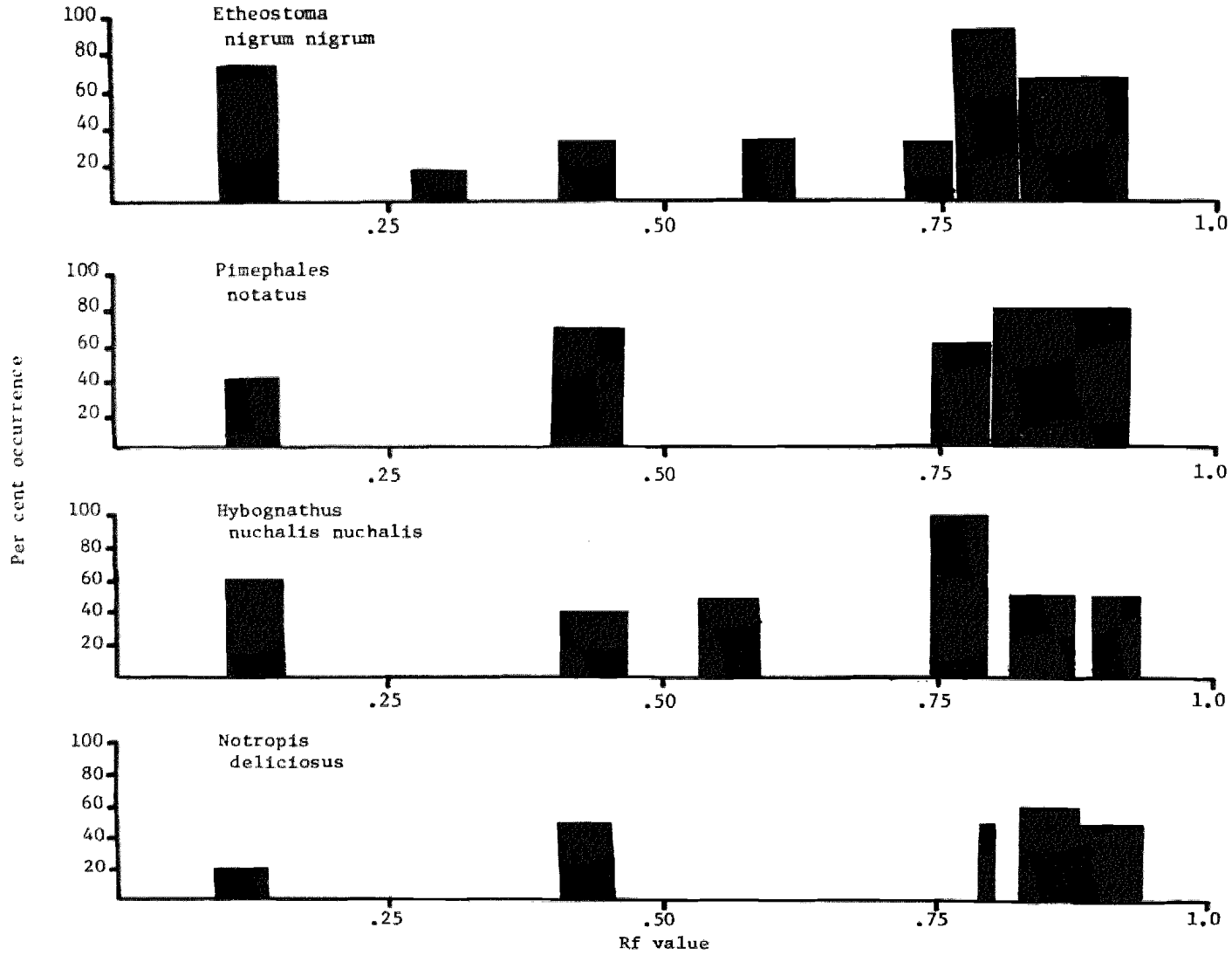


Figure V The per cent occurrence values of the spots found on chromatograms of Etheostoma nigrum nigrum, Pimephales notatus, Hybognathus nuchalis nuchalis and Notropis deliciosus.

Comparison of chromatograms of separate families, Percidae and Cyprinidae, showed that distinct differences existed in their body mucus. When comparing chromatograms of three genera, Hybognathus, Pimephales and Notropis of the family Cyprinidae fewer differences were found than between the two families. The genera, however could be distinguished from one another, by either the number and location of spots or by the general appearance of their chromatograms. Comparisons of two closely related species showed identical chromatograms. The chromatograms of Notropis deliciosus and Notropis dorsalis dorsalis showed no significant difference in the number and location of spots. Differences were observed in the per cent occurrence of comparable spots. Illustrating this best are the chromatograms of figure II and the per cent occurrence graph of figure III. Here it is observed that the spot with the lowest Rf value occurs in only 6.25% of the Notropis deliciosus chromatograms and in 87.0% of the Notropis dorsalis dorsalis chromatograms. A possible explanation of this and of the fact that in the preliminary studies no carbohydrate element was found, which contrasts with the findings of Wessler and Werner (1957) and Huntsman (1964), is that quantitative differences occur in the components of fish body mucus along with qualitative differences. Since no observable differences could be shown in the chromatograms of two closely related species it is possible that only quantitative differences exist. Carlson (1961) found this to be the case when

working with amino acids of lamprey muscle. It is questionable that quantitative differences alone could account for the variation in per cent occurrence values of comparable spots. However this fact and the small and variable amount of mucus used for each chromatogram combined, could be enough to cause the differences. In a verbal communication with Holger W. Jannasch he stated that in his work he had trouble picking up bacterial metabolities by chromatographic techniques. He thought there was a lower limit of detectability or that a minimum quantity of a sample is needed before separation and identification can take place. One must also remember that the factors effecting the color development of ninhydrin stained chromatograms will influence this variability.

On the original object of this study, the species specificity of fish body mucus, it must be stated that closely related species seem to have similar if not identical body mucus composition, except for possible quantitative differences. To prove this positive quantitative analysis of mucus will have to be accomplished. Until this is done it would seem, at least for certain groups of closely related species, that separation of species cannot be attained simply through the use of chromatography. Huntsman (1964) also found similar chromatograms, which could not separate closely related species. Barry and O'Rourke (1959), on the other

hand, stated they separated species of the genus Gadus. It could be then that a very specific system needs to be developed for the different groups of fish.

The method of chromatography was found to give quite variable results. This can be seen at least in part on the per cent occurrence graphs, figures III and V. Here one can see in what per cent of the chromatograms a certain spot showed up. The majority of the identifiable spots showed up on a minimum of 50% of the chromatograms. The variations can be caused by environmental conditions, such as humidity and temperature by themselves. With these factors constantly being present and constantly changing, variations will occur in chromatograms. Constant laboratory conditions would limit these variations and thus improve the method. This plus ever improving chromatographic methods leaves chromatography as a useful taxonomic tool, now and in the future. Other chemical methods of defining a species seem to be just as good if not better than chromatography. Serological methods and electrophoresis have been used and show promise of being very useful in taxonomic work.

SUMMARY

The taxonomy of any group of organisms goes through various stages of sophistication. One must remember, however, that new methods are developed not to replace, but to

supplement previous methods (Mayr, 1964). This study is essentially one in the field of comparative biochemistry. When working in this field one looks for similarities and differences of composition and reaction in the phylogenetic system. One of the most widely examined compounds is the proteins. It is accepted that the amino acid sequence of proteins represents the expression of genetic information (Florkin, 1964). Anfinsen proposed the concept of protein spectrum, which states, "Since proteins can be modified without loss of function, it seems certain that the permissible degree of modifications in terms of fractions of their total structure, will vary somewhat from molecular species to species". At first genetics appeared the answer to classification problems. It was discovered, however, that phenotypes only partially represent genetic differences. A single gene can show two entirely different phenotypes. The same phenotype can also be acquired through different routes. These factors have dampened the optimism about genetics and its usefulness in taxonomy. Nevertheless although genetics isn't a complete answer to classification, it is the basis for most work (Mayr, 1964).

In this study ITLC proved to have a somewhat limited use in taxonomy when applied to body mucus. Chromatograms gave individual characteristic patterns associated with family and genera differences. The differences between families

were much more prounounced than those between genera. When closely related species were compared, no differences could be seen. Even though differences between certain taxonomic groups were observed, they were found by developing many chromatograms of each species. Inconsistencies occurred in many of the individual chromatograms; thus to find one specimen and attempt to classify it on the basis of one chromatogram would be very questionable. This seems to be the case for not only ITLC but also for chromatography in general. Huntsman (1964) found similar inconsistencies in chromatograms when working with fish body mucus. These inconsistencies were probably due to the many environmental conditions which effect the running of chromatograms and their development. If laboratory conditions cannot be kept stable these inconsistencies will occur and hinder the use of chromatography in taxonomy. Perhaps with improvements in methodology, chromatography will become a more useful tool for taxonomy.

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